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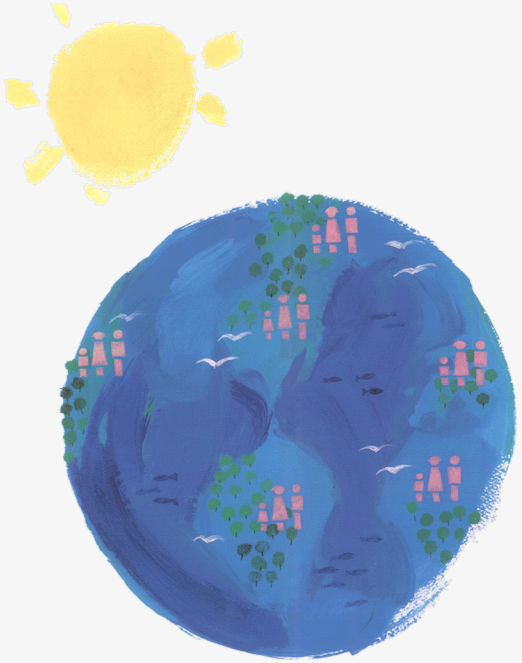
National Toxicology Program
Interagency Center for the Evaluation of Alternative Toxicological Methods

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods



Independent Peer Review Panel Report: Five *In Vitro* Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products

Karen Brown, Ph.D.
Chair, *In Vitro* Pyrogenicity Peer Review Panel

June 12, 2007
SACATM Meeting
Marriott Bethesda North Conference Center
Bethesda, MD

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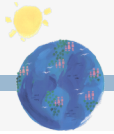
ICCVAM Charges to the Peer Panel

- Review the ICCVAM draft In Vitro Pyrogen Test Methods Background Review Document (BRD) for completeness, and identify any errors or omissions in the BRD
- Evaluate the information in the draft BRD to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods have been appropriately addressed
- Consider the ICCVAM draft test method recommendations for the following and comment on the extent to which are supported by the information provided in the BRD
  - Proposed test method use
  - Proposed recommended standardized protocols
  - Proposed test method performance standards
  - Proposed future studies

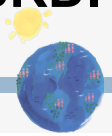
Peer Review of the ICCVAM BRD for Completeness, Errors, and Omissions (1)

- In general, the Panel considered the information presented in the ICCVAM draft BRD to be sufficient for its purpose.
  - Provides a comprehensive review of available data and information regarding the usefulness and limitations of the in vitro pyrogen test methods
  - Provides a description of the current validation status of the in vitro pyrogen test methods



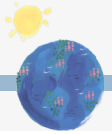
Peer Review of the ICCVAM BRD for Completeness, Errors, and Omissions (2)

- The Panel identified a number of sections where clarification or a more comprehensive explanation would improve the ICCVAM draft BRD. For example:
  - The extent to which the RPT is currently performed when risk assessments and regulatory decisions are concerned only with the presence of endotoxin should be provided.
  - The number of rabbits used for pyrogenicity testing should be provided to permit an accurate assessment of the actual impact on animal use.
  - The cost and logistical considerations involved in conducting a study using the in vitro test methods were incompletely stated.
  - Both the cost and logistical problems associated with the need to harvest and use human blood in four of the test methods were understated.
- The Panel also requested that the formal validation statement from the ECVAM Scientific Advisory Committee (ESAC) (and the supporting documents) be appended to the ICCVAM BRD.



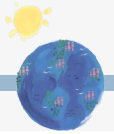
List of Substances Tested

- **The rationale for the selected test substances was neither appropriate nor acceptable.**
  - **Only rationale is that the substances were manufactured under GMP, were licensed products, were reported not to be contaminated with unacceptable levels of endotoxin, and were all available at reasonable cost.**
  - **According to their USP monographs, seven of the ten test substances are currently tested in the Bacterial Endotoxin Test (BET), not in the Rabbit Pyrogen Test (RPT).**
    - **No USP monographs exist for the remaining three because pyrogen testing is not required.**
- **Non-endotoxin pyrogens, protein- and lipid-containing materials that are used parenterally, and 'classical' examples of biological products and medical devices should have been included.**
- **The total number of substances included in the validation study (n=10) is adequate only for validation of a specific class of products.**
  - **Replacement of the RPT would require a much larger number of substances because of the wide range of product classes that would require testing.**



In Vivo Reference Data

- The reference data were previously and separately generated by one protocol, in one laboratory, using one strain of rabbit, and two sources of endotoxin.
- The lack of direct parallel testing in rabbits with the products tested in the validation study was a significant limitation to the study design.

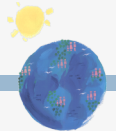


Test Method Relevance (1)

- **The evaluation of relevance (i.e., concordance, sensitivity, specificity, positive and negative predictivity, false positive and negative rates) appears to have been appropriately demonstrated and discussed, but limited by the ability to judge a positive versus negative response using a cut-off at 0.5 endotoxin units (EU)/mL.**
- **Because only endotoxin-spiked samples were tested, relevance has been demonstrated only for the detection of bacterial endotoxin.**
- **This section is entirely focused on comparisons between the in vitro pyrogen test methods since the RPT was not carried out in parallel.**
  - Estimates of the RPT performance were modeled statistically.
  - Therefore, no data exist with which to establish concordance with the RPT and thus, the discussion on concordance with the RPT is speculative.

Test Method Relevance (2)

- Inadequate performance is noted for:
  - Cryo WB/IL-1 (false positive rate = 18.1%)
  - WB/IL-1 (false negative rate = 27.3%)
  - WB/IL-1 (false positive rate = 16.4%).
- These statements could indicate that the WB/IL-1 assays (WB/IL-1 Cryo WB/IL-1, and WB/IL-1 96-well plate method) do not, in general, perform as well as the other assays that measure an IL-6 response.
- It would have been very interesting to have had the opportunity to compare performance analysis data for the BET, since only endotoxin spiked samples were used in the validation and endotoxin testing is now the intended use for the in vitro pyrogen tests.
  - Unfortunately, the BET was not performed in the validation so no direct comparison can be made between it and the new in vitro assays.

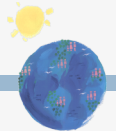


Test Method Reliability (1)

- **The analyses based on 'positive or negative' calls suggests that the reliability of these in vitro test methods are generally acceptable both within and between laboratories, although a more critical description is needed to explain the lack of agreement among some test results.**
- **A quantitative assessment of the intra- and inter-laboratory variability would have been more informative than an assessment based on dichotomizing the test results.**
  - The assessment should have included estimates of the amount of inter- and intra-laboratory variability and the number of replicates needed to estimate the sources of variability.
  - Acceptable levels of variability should have been identified *a priori*, and it should have been recognized that formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

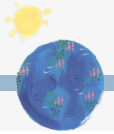
Test Method Reliability (2)

- **Deficiencies noted include:**
  - **The high exclusion rate for individual runs in the case of the Cryo WB/IL-1 test (20% - 30% out of 150 runs) due to excessive variability among the four replicates, even with a relatively high coefficient of variation (CV) criteria (CV > 45%).**
  - **Agreement across three validation laboratories was only 57% for the WB/IL-1 assay.**
  - **It would have been more appropriate to evaluate reliability using a subset of the drugs used in the sensitivity/specificity studies.**



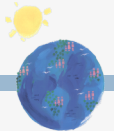
Validation Status of the *In Vitro* Pyrogen Test Methods (1)

- **The Panel agreed that the applicable validation criteria have been adequately addressed in the ICCVAM draft BRD in order to determine the usefulness and limitations of these test methods to serve as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.**
- **Minority opinions:**
  - **The qualification in the above statement (i.e., that uses were subject to product specific validation) should allow for these test methods to be used for the specified purpose (Dr. Peter Theran).**
  - **It is not clear that the qualification included in the above statement would preclude the use of the in vitro test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT (Drs. Karen Brown, Albert Li, and Jon Richmond).**



Validation Status of the *In Vitro* Pyrogen Test Methods (2)

- **However, the Panel generally agreed that the performance of these test methods in terms of their reliability and relevance did not support this proposed use (i.e., as a substitute for the RPT, for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation).**
- **Minority opinions:**
  - **The qualification in the above statement (i.e., that uses were subject to product specific validation) should allow for these test methods to be used for the specified purpose (Dr. Peter Theran).**
  - **It is not clear that the qualification included in the above statement would preclude the use of the in vitro test methods as replacements for the RPT in those circumstances where the BET is currently serving to replace the RPT (Drs. Karen Brown, Albert Li, and Jon Richmond).**



- **Does the Panel agree that the available data and demonstrated performance in terms of relevance and reliability support the ICCVAM draft recommendations for these *in vitro* test methods in terms of the:**
  - **Proposed test method usefulness and limitations**
  - **Proposed test method standardized protocols**
  - **Proposed test method performance standards**
  - **Proposed additional studies**

Draft ICCVAM Proposed Test Method Usefulness and Limitations

- ***Panel Response:*** **Not supported by the BRD, for the following reasons:**
  - **Usefulness of test methods for detection of Gram-negative endotoxin was not properly assessed for concordance with RPT nor for relevance in comparison to the BET.**
  - **Failure to evaluate non-endotoxin pyrogens in the validation study limited evaluation of the practical usefulness and limitations of these test methods - should be included in future efforts.**
  - **Test materials in pure form may stimulate cytokine production limiting usefulness.**
  - **However, the Panel does recognize that mechanisms exist for test method development on a case-by-case basis subject to product-specific validation to demonstrate equivalence to the RPT (21CFR 610.9).**
  - **Minority Opinion (Dr. Peter Theran): Recommendations for de novo rabbit testing should be accompanied by the following statement: "The use of rabbits in new parallel tests for the validation of an in vitro test should only be conducted after a vigorous search for a scientifically sound, non-animal alternative (i.e., the need for additional animal studies must be justified on a case-by-case basis)."**

Draft ICCVAM Proposed Standardized Test Method Protocols

- ***Panel Response:*** **Yes, supported by the BRD if the list of inadequacies are fully addressed. For example:**
  - **Use multiple donors with similar acceptance criteria and exclusion rules for each test method to reduce variability.**
  - **"Benchmark" test design is preferred to "limit" test design based on i.v. fever threshold.**
  - **Specify in more detail blood donor recruitment and selection criteria and conditions for venipuncture.**

Draft ICCVAM Proposed Test Method Performance Standards

- *Panel Response:* Not supported by the BRD, based on the following inadequacies:
  - **Essential Test Method Components**
    - **Define adequately stringent CV criterion**
    - **Define number of donors used in a pool**
  - **Accuracy and Reliability Values**
    - **Two assays have false positive rates greater than 16%.**
    - **If intended use of in vitro test methods is restricted to detection of endotoxin, they should be compared to both the BET and to the RPT, since the BET is currently used in lieu of the RPT for this purpose.**
  - **Minimum List of Reference Substances**
    - **For consideration as replacement for the RPT, the in vitro test methods must be validated for all classes of substances and for endotoxin and non-endotoxin pyrogens.**
  - **Minority Opinion (Dr. Peter Theran):** Identical to that expressed for proposed test method usefulness and limitations

Draft ICCVAM Proposed Additional Studies

- **The Panel agrees that to better determine the potential of these test methods, the proposed additional studies should be performed, taking into account the comments and recommendations detailed previously.**
- **Additional Panel recommendations include:**
  - **Establish a repository of clinically-identified pyrogens for use in future validation studies**
  - **Test both endotoxin and non-endotoxin pyrogens in future validation studies.**
  - **Prospectively compare in vitro tests with RPT and BET in future validation studies**
  - **Evaluate the correlation of IL-1 and IL-6 levels in the in vitro tests with levels produced in rabbits using similar doses of endotoxin**
  - **Minority Opinion (Dr. Peter Theran): Identical to that expressed for proposed test method usefulness and limitations**

Overall Peer Review Conclusions: Future Potential for the *In Vitro* Pyrogen Tests

- ***These test methods could be applicable to a wider range of pyrogens and test materials, provided that they are adequately validated for such uses.***
- ***It is critical to recognize, despite concerns about the performance of these five in vitro test methods, that a formal process exists for materials regulated under 21 CFR 610.9 to qualify these in vitro methods for the identification of Gram-negative endotoxin on a case-by-case basis, subject to product specific validation.***

ICCVAM *In Vitro* Pyrogenicity Peer Panel

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  Eli Lilly
  Indianapolis, Indianapolis

Discussion Questions for SACATM

(Lead Discussants: Drs. Barile, McClellan, Qu)

1. **Do you have any comments on the panel's conclusions and recommendations on the draft ICCVAM Background Review Document (BRD) in regard to its completeness and any identified errors or omissions?**
2. **Do you have any comments on the panel's conclusions and recommendations in terms of the extent to which each of ICCVAM's applicable criteria for validation and acceptance of toxicological test methods have been addressed appropriately in the BRD?**
3. **Do you have any comments on the draft ICCVAM test method recommendations for the five in vitro pyrogenicity test methods?**
4. **Do you have any comments on the panel's comments conclusions on the draft ICCVAM test method recommendations for the five in vitro pyrogenicity test methods regarding**
   a. **their usefulness and limitations**
   b. **the recommended test method protocols**
   c. **test method performance standards**
   d. **the proposed additional studies**